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OUTSTANDING OBSERVATION

An instructive role of donor macrophages in mixed chimeras in the induction of recipient CD4$^+$Foxp3$^+$ Treg cells

Guangwei Liu, Kaizhong Duan, Haixia Ma, Zeqing Niu, Jianxia Peng and Yong Zhao

The immune regulatory function of macrophages (Møs) in mixed chimeras has not been determined. In the present study, with a multi-lineage B6-to-BALB/c mixed chimeric model, we examined the ability of donor-derived splenic Møs in the induction of regulatory T cells (Treg). B6 splenic Møs from mixed chimeras induced significantly less cell proliferation, more IL-10 and TGF-β, and less IL-2 and IFN-γ productions of CD4$^+$ T cells from BALB/c mice than naive B6 Møs did, whereas they showed similar stimulatory activity to the third part C3H CD4$^+$ T cells. Importantly, highly purified donor F4/80$^+$CD11c$^-$ Møs efficiently induced recipient CD4$^+$Foxp3$^+$ Treg cells from CD4$^+$CD25$^-$Foxp3$^-$ T cells. Furthermore, donor Møs of mixed chimeras produced more IL-10 and less IFN-γ than those of naive mice when cultured with BALB/c but not the third party C3H CD4$^+$ T cells. Induction of recipient CD4$^+$ Treg cells by donor Møs was significantly blocked by anti-IL-10, but not by anti-TGF-β mAb. Therefore, donor Møs have the ability to induce recipient CD4$^+$Foxp3$^+$ Treg cells in a donor antigen-specific manner, at least partially, via an IL-10-dependent pathway. This study for the first time showed that, in mixed allogeneic chimeras, donor Møs could be specifically tolerant to recipients and gained the ability to induce recipient but not the third party Foxp3$^+$ Treg cells. Whether this approach is involved in transplant immune tolerance needs to be determined.
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Mononuclear phagocytes, an important part of innate immunity, have pivotal roles in pathogen and tissue debris clearance, antigen capture and presentation, as well as in shaping the development of adaptive immune response. Resident macrophage (Mø) populations in different organs adapt to their local microenvironments,[1] so that they display a wide diversity in terms of their function and morphology.[2] The heterogeneity of Møs may be important for the diversity, flexibility and validity of innate and adaptive immunity to all kinds of stimulus.[3] Recently, Møs have been implicated in suppression of autoreactive T cells and in controlling CD4$^+$ T-cell hyporesponsiveness, indicating that Møs may also have an active role in inducing/maintaining immune tolerance toward self-antigens.[4]

Studies have shown that Møs, which were differentiated from myeloid precursors, are involved in the acute and chronic rejection of allografts or xenografts.[5–9] In mixed allogeneic chimeric mouse models, donor and recipient T, B and NK cells are well defined to be tolerant to both recipient and donor antigens.[10] Donor antigen-presenting cells including Møs are closely involved in the negative selection of alloreactive T cells in the thymus to achieve T-cell tolerance to alloantigens in these models.[10] Recently, regulatory CD4$^+$CD25$^+$ T cells (Treg) expressing a unique transcription factor Foxp3 have been demonstrated to have an important role in control immune response or tolerance to self-antigens, tumor antigens, transplant antigens or pathogens.[11–12] It has been reported that certain subset or stage of Møs or dendritic cells (DCs) have the potential ability to induce CD4$^+$CD25$^+$ Treg cells or mediate immune tolerance induction.[13] Møs in mixed chimeras showed normal immunity including phagocytosis and antigen-presenting ability.[14] However, whether donor Møs in mixed chimeras have the ability to induce Treg cells in periphery has not been addressed. Our present study showed that donor C57BL/6 (B6) splenic Møs from mixed chimeras have the ability to induce antigen-specific CD4$^+$Foxp3$^+$ Treg cells from CD4$^+$CD25$^-$Foxp3$^-$ T effector cells of naive BALB/c mice but not from the third party CD4$^+$CD25$^-$Foxp3$^-$ T effector cells, at least partially, via an IL-10-dependent pathway. Thus, Møs in mixed chimeras were tolerant to donor and host antigens as evidenced by induction of CD4$^+$Foxp3$^+$ Treg cells in an unknown antigen-specific manner. The present study offers evidence for the specific tolerance of Møs. Whether the gained ability of Møs to induce CD4$^+$Foxp3$^+$ Treg

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cells is involved in transplant immune tolerance and possibly also in self-tolerance, requires to be determined.

RESULTS

Decreased immunogenicity of donor B6 Møs from B6-to-BALB/c mixed chimeras to CD4$^+$ T cells from naive BALB/c recipients not the third party C3H mice

The phenotype of B6 F4/80$^+$ splenic Møs in B6-to-BALB/c mixed chimeras was detected by flow cytometry (FCM) at about 10 weeks after bone marrow transplantation. Significantly, less percentages of donor B6 F4/80$^+$Møs separated from chimeras express MHC II, CD86 and CD40, compared with those from naive B6 mice (Supplementary Figure 1). However, identical levels of MHC II, CD80, CD86, CD40, CD54 and CD23 expressions on B6 F4/80$^+$Møs were observed after lipopolysaccharide stimulation *in vitro*, regardless they were separated from mixed chimeras and B6 mice (Supplementary Figure 1).

We first determined the stimulatory activities of donor Møs prepared from mixed chimeric mice *in vitro*. As expected, B6 Møs efficiently stimulated the proliferation of C3H CD4$^+$ T cells as assayed by ^{3}H-TdR incorporation, regardless of whether they were separated from control or mixed chimeric mice (Figure 1A). However, B6 Møs from mixed chimeras induced significantly lower proliferation of CD4$^+$ T cells from naive BALB/c mice compared with those Møs from naive control B6 mice, suggesting a reduced ability to efficiently trigger the proliferation of recipient BALB/c but not the third party C3H CD4$^+$ T cells (Figure 1A). The decreased proliferation of CD4$^+$ T cells from naive BALB/c mice induced by B6 Møs from mixed chimeras was consistently observed in different ratios between B6 F4/80$^+$Møs stimulators and BALB/c T-cell responders (Figure 1B). On the other hand, recipient BALB/c Møs separated from mixed chimeras showed markedly decreased immunogenicity to T cells of naive donor B6 mice, but not to the third party C3H mouse T cells compared with BALB/c Møs from naive mice (data not shown). Thus, Møs from mixed chimeras showed somewhat tolerant characteristics to both donor and host antigens in an antigen-specific manner as indicated by the decreased ability to promote T-cell response.

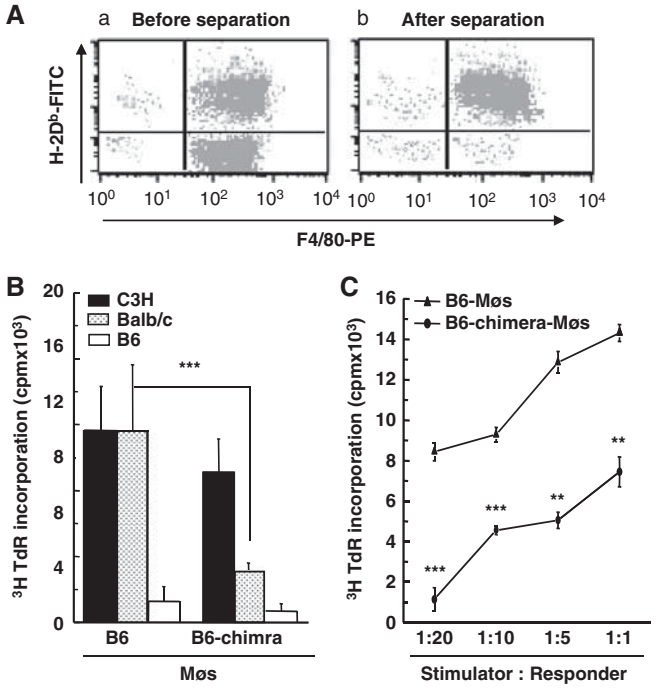

Figure 1 Markedly reduced immunogenicity to recipient CD4$^+$ T cells of donor splenic Møs separated from mixed chimeras. (**A**) The purity of donor B6 splenic Møs before and after MACS separation. (a) Adhesive cells before separation. (b) Purified donor splenic Møs after MACS separation. More than 90% of cells were H-2D^{b+}and F4/80$^+$. (**B**) Donor B6 splenic Møs separated from B6-to-BALB/c mixed chimeras showed significantly reduced stimulating ability to CD4$^+$ T cells from either naive recipient BALB/c, but not the third party C3H mice, when assayed by ^{3}H-thymidine incorporation *in vitro*. (**C**) Donor B6 splenic Møs from mixed chimera induced significantly lower proliferation of CD4$^+$ T cells from naive recipient BALB/c mice than B6 splenic Møs among different ratios between Møs and CD4$^+$ T cells. $^{**}P<0.01$ and $^{***}P<0.001$ between the indicated groups. Seven mice in each group were done. Data are shown as mean ± s.d. One representative of three independent experiments with similar results was shown.

Recipient CD4$^+$ Treg cells could be induced efficiently by donor Møs of mixed chimeras

We next investigated whether Møs separated from mixed chimera have the ability to induce Treg cells. As shown in Figure 2, after co-culture with B6 splenic Møs separated from B6-to-BALB/c mixed chimeras, CD4$^+$ T cells of naive BALB/c mice significantly inhibited the BALB/c CD4$^+$ T-cell proliferation induced by allogeneic B6 antigens in a dose-dependent manner *in vitro* (Figures 2a and b), whereas BALB/c CD4$^+$ T cells pre-stimulated with normal B6 Møs did not show detectable immunosuppressive functions. The third party C3H CD4$^+$ T cells stimulated with B6 Møs from either naive B6 or mixed chimeras did not have detectable inhibitory effects on C3H CD4$^+$ T effector cells (Figure 2a). In addition, IL-2 production of BALB/c T effector cells was significantly inhibited by donor B6 Møs-induced BALB/c Treg cells in primary mixed lymphocyte reaction (MLR; $P<0.001$; Figure 2c).

Recipient CD4$^+$ Treg cells induced by donor Møs of mixed chimeras are antigen specific

The antigen specificity of CD4$^+$ Treg cells induced by donor Møs of mixed chimeras was determined *in vitro*. As shown in Supplementary Figure 2, induced recipient BALB/c CD4$^+$ Treg cells by donor Møs from B6-to-BALB/c mixed chimeras significantly inhibited the proliferation of BALB/c CD4$^+$ T effector cells induced by donor B6 antigens, but not by the third party C3H antigens (Supplementary Figure 2). These results demonstrate that recipient CD4$^+$ Treg cells induced by donor Møs of mixed chimeras have donor antigen specificity.

Recipient CD4$^+$ Treg cells induced by donor splenic Møs of mixed chimeras express Foxp3

It is well known that Foxp3 is a specific transcription factor in CD4$^+$CD25$^+$ Treg cells in mice.[15–16] Thus, the expression of Foxp3 in the induced CD4$^+$ Treg cells in the present model was analyzed by a three-color FCM. After CD4$^+$ T cells from naive BALB/c mice were cultured with donor B6 Møs separated from B6-to-BALB/c mixed chimeras, significantly more cells of recipient BALB/c CD4$^+$ T cells, CD4$^+$CD25$^+$ T cells and even CD4$^+$CD25$^-$ T cells expressed high levels of Foxp3 than those stimulated with naive B6 Møs, as determined by FCM (Figures 3A and B). It is noted that there is significantly higher percentage of Foxp3$^+$ cells in CD4$^+$CD25$^+$ T-cell fraction than those in CD4$^+$CD25$^-$ T-cell fraction after CD4$^+$ T cells from naive BALB/c mice cultured with donor B6 splenic Møs separated from B6-to-BALB/c mixed chimeras (Figures 3B, b and c). Furthermore, after culture with donor B6 splenic Møs from mixed chimeras, CD4$^+$ T cells from naive BALB/c mice produced significantly more numbers of CD4$^+$Foxp3$^+$ T cells, CD4$^+$CD25$^+$Foxp3$^+$

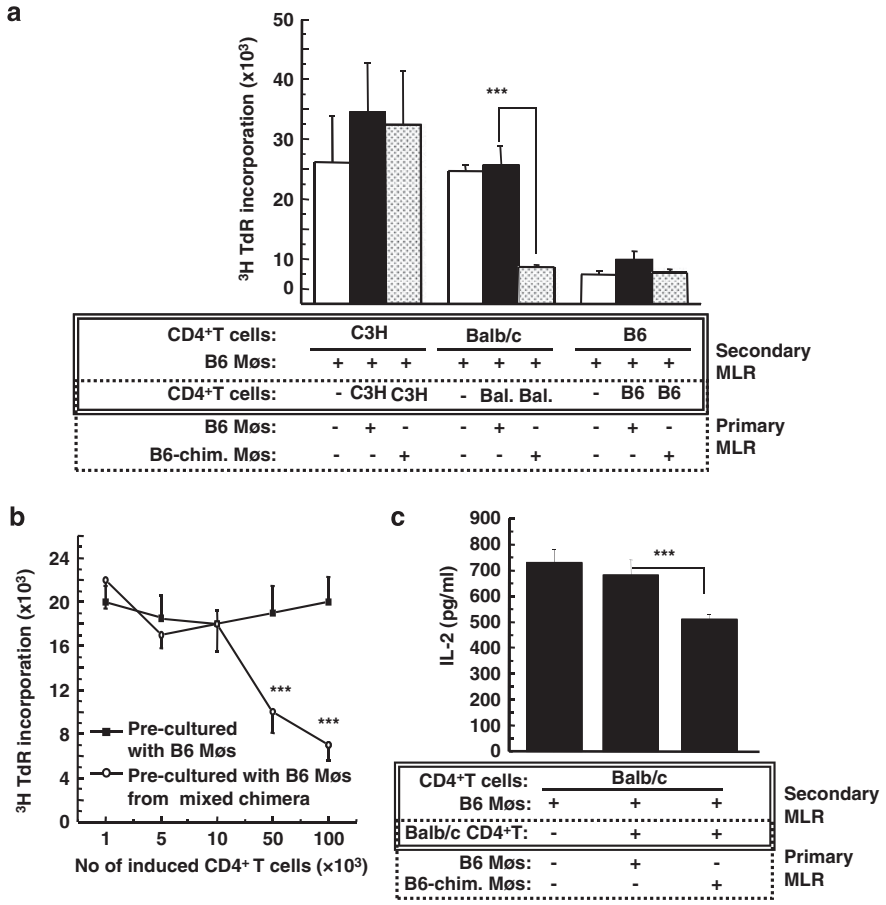

Figure 2 Recipient but not the third party CD4$^+$ Treg cells were induced by donor splenic Møs of mixed chimeras *in vitro*. (**a**) CD4$^+$ T cells from naive BALB/c mice showed immunosuppressive function after co-culture with donor B6 splenic Møs separated from B6-to-BALB/c mixed chimeras *in vitro*. CD4$^+$ T cells of naive B6, BALB/c or C3H mice were cultured with B6 splenic Møs from control or mixed chimeras for 4–5 days, respectively. These pre-cultured CD4$^+$ T cells were then added to the secondary culture systems in which B6, BALB/c or C3H CD4$^+$ T cells were cultured with normal B6 Møs stimulators. (**b**) The inhibiting ability of naive BALB/c CD4$^+$ T cells pre-cultured with B6 splenic Møs of mixed chimeras is dose dependent. (**c**) The inhibition on IL-2 products of T effector cells by non-chimera BALB/c CD4$^+$ T cells pre-cultured with B6 splenic Møs of mixed chimeras. ***$P<0.001$ between the indicated groups. Seven mice in each group were performed. Data are one representative of three independent experiments. Results are shown as mean ± s.d.

T cells and CD4$^+$CD25$^-$Foxp3$^+$ T cells than those stimulated with naive B6 splenic Møs (Figure 3C). These data indicate that donor splenic Møs from mixed chimeras could induce Foxp3$^+$ Treg cells and that the induced recipient CD4$^+$Foxp3$^+$ Treg cells predominately presented in CD4$^+$CD25$^+$ and some in CD4$^+$CD25$^-$ T-cell fractions.

Recipient CD4$^+$Foxp3$^+$ Treg cells induced by donor splenic Møs of mixed chimeras were derived from CD4$^+$CD25$^-$Foxp3$^-$ T cells

To determine whether recipient CD4$^+$ Treg cells induced by donor Møs from mixed chimeras are due to the differentiation of CD4$^+$CD25$^-$ T cells or the expansion of CD4$^+$CD25$^+$ Treg cells, we studied the effects of donor Møs on recipient CD4$^+$CD25$^-$ T cells. The sorted CD4$^+$CD25$^-$ T cells of naive BALB/c mice did not express Foxp3 and their purity was always >99% (Supplementary Figure 3a). BALB/c CD4$^+$CD25$^-$ T cells expressed CD25, no matter whether they were cultured with donor B6 Møs separated from B6-to-BALB/c mixed chimeras or from naive B6 mice (Supplementary Figures 3b and c). The total cell numbers of CD4$^+$ T cells and CD4$^+$CD25$^+$ T cells after culturing CD4$^+$CD25$^-$ T cells with B6 Møs from mixed chimeras were significantly lower than those cultured with B6 Møs from naive B6 mice (Supplementary Figure 3d). However, remarkably higher percentages and more cell numbers of CD4$^+$Foxp3$^+$ T cells, CD4$^+$CD25$^+$Foxp3$^+$ T cells and CD4$^+$CD25$^-$Foxp3$^+$ T cells were observed when BALB/c CD4$^+$CD25$^-$ T cells were cultured with B6 Møs from B6-to-BALB/c mixed chimeras compared with BALB/c CD4$^+$CD25$^-$ T cells cultured with B6 Møs from non-chimeras (Supplementary Figures 3c and d). To confirm the immunosuppression of the induced CD4$^+$Foxp3$^+$ Treg cells, we observed their inhibitory effects on the response of T effector cells to alloantigens. BALB/c CD4$^+$CD25$^+$ Treg cells induced by B6 Møs of mixed chimeras showed similar inhibition effects on effector T cells as freshly isolated BALB/c CD4$^+$CD25$^+$ Treg cells (Supplementary Figure 3e). Thus, donor Møs in mixed chimeras have the ability to convert recipient CD4$^+$CD25$^-$Foxp3$^-$ T cells into CD4$^+$Foxp3$^+$ Treg cells.

To further confirm whether the isolated donor Møs or some contaminated DCs have the ability to induce Treg cells, and whether the induced Treg cells are derived from CD4$^+$Foxp3$^-$ T effector cells, we used highly purified H-2D^{b+}F4/80$^+$CD11c$^-$ cells and CD4$^+$Foxp3$^-$ cells sorted by a sorter, instead of magnetic activated cell sorting (MACS)-enriched Møs and CD4$^+$CD25$^-$ cells, to perform the similar experiments. As shown in Figure 4, >99% of sorted B6 Møs cells were

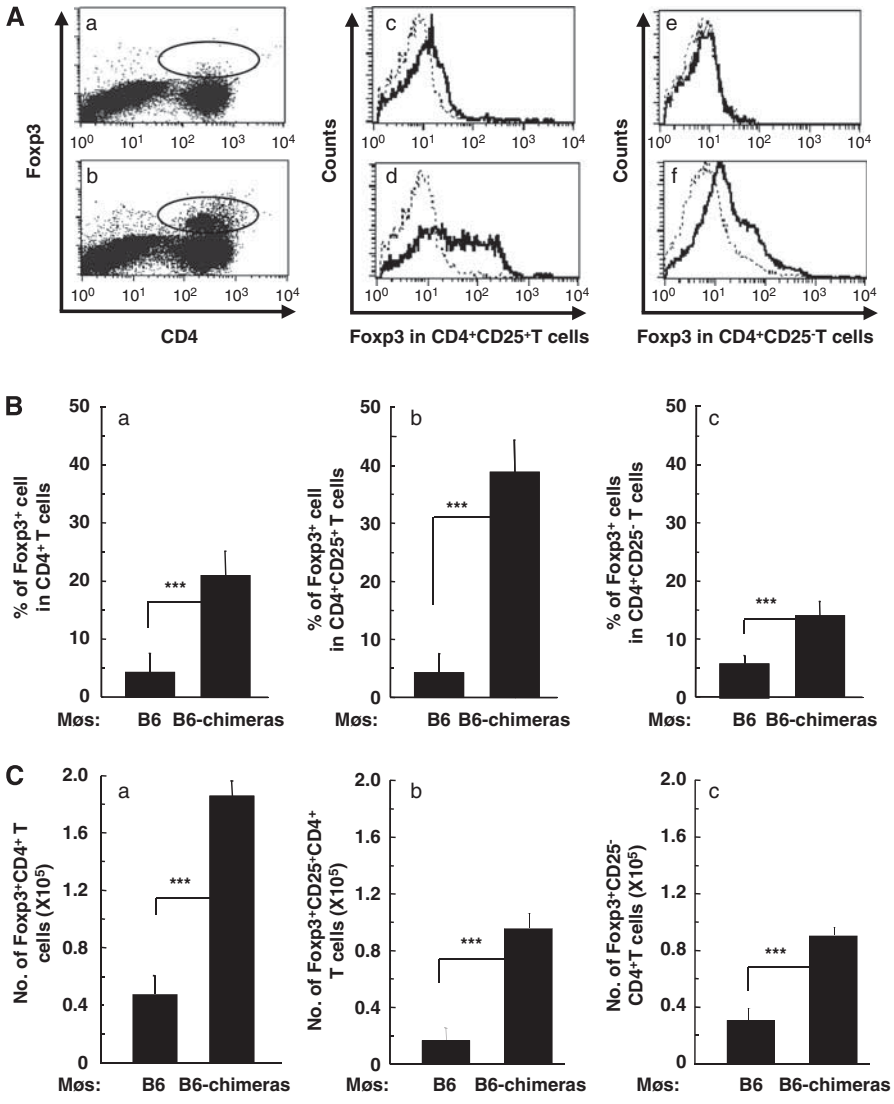

Figure 3 Donor Møs-induced allogeneic recipient CD4$^+$ Treg cells express Foxp3. (**A**) After culture with B6 splenic Møs from mixed chimeras or control mice, the expression CD25 and Foxp3 on recipient BALB/c CD4$^+$ T cells was determined by three-color FCM. One representative of the expression of CD25 and Foxp3 on CD4$^+$ T cells was shown as detected by three-color FCM. CD4$^+$ T cells from naive BALB/c mice were cultured with B6 splenic Møs from non-chimeras (a, c and e) or from B6-to-BALB/c mixed chimeras (b, d and f). The dotted line represents the non-specific staining and the solid line is for Foxp3 staining. (**B**). The mean percentages of Foxp3$^+$ cells in CD4$^+$ T cells (a), CD4$^+$CD25$^+$ T cells (b) or CD4$^+$CD25$^-$ T cells (c) after naive BALB/c CD4$^+$ T cells were cultured with donor B6 splenic Møs from mixed chimeric mice or naive B6 mice. (**C**) The total cell numbers of BALB/c CD4$^+$Foxp3$^+$ T cells (a), CD4$^+$CD25$^+$Foxp3$^+$ T cells (b) and CD4$^+$CD25$^-$Foxp3$^+$ T cells (c) after BALB/c CD4$^+$ T cells stimulated with B6 splenic Møs from mixed chimeras or naive B6 mice. ***$P<0.001$ between the indicated groups. One representative from five independent experiments with identical results is shown. Data are shown as mean ± s.d.

H-2D^{b+}F4/80$^+$CD11c$^-$ (Figure 4a). These purified B6 H-2D^{b+}F4/80$^+$CD11c$^-$ Møs isolated from mixed chimera showed poor immunogenicity to recipient T cells ($P<0.001$), while they induced strong proliferation of the third party C3H T cells (Figure 4b). Consistently, the purified B6 H-2D^{b+}F4/80$^+$CD11c$^-$ Møs isolated from mixed chimera induced significantly more CD4$^+$Foxp3GFP$^+$ T cells when they were cultured with recipient CD4$^+$Foxp3GFP$^-$ T effector cells, compared with control B6 Møs ($P<0.001$; Figures 4c–e). Furthermore, the induced CD4$^+$Foxp3GFP$^+$ T cells significantly inhibited the proliferation of naive CD4$^+$Foxp3GFP$^-$ T effector cells. Thus, these data further support that donor F4/80$^+$CD11c$^-$ Møs in allogeneic mixed chimera have the ability to induce the differentiation of recipient CD4$^+$Foxp3$^+$ T cells from CD4$^+$Foxp3$^-$ T effector cells.

The induction of recipient CD4$^+$ Treg cells by donor Møs mixed chimeras is partially mediated by IL-10, but not by TGF-β

To determine whether cytokines produced by donor splenic Møs of mixed chimeras is involved in the induction of CD4$^+$Foxp3$^+$ Treg cells, we assayed the cytokine profile of donor B6 Møs from mixed chimeras during culture with CD4$^+$ T cells from either naive recipient BALB/c or the third party mice. As shown in Supplementary Figure 4, after culture with BALB/c CD4$^+$ T cells, B6 Møs from B6-to-BALB/c mixed chimeras expressed significantly higher levels of IL-10 than those of B6 Møs from naive B6 mice as determined by intracellular staining FCM ($P<0.01$, $P<0.001$, respectively; Supplementary Figures 4a and b), whereas similar percentages of IL-10$^+$ or IFN-γ^+ Møs were observed in B6 Møs separated either from mixed chimeras or from naive mice

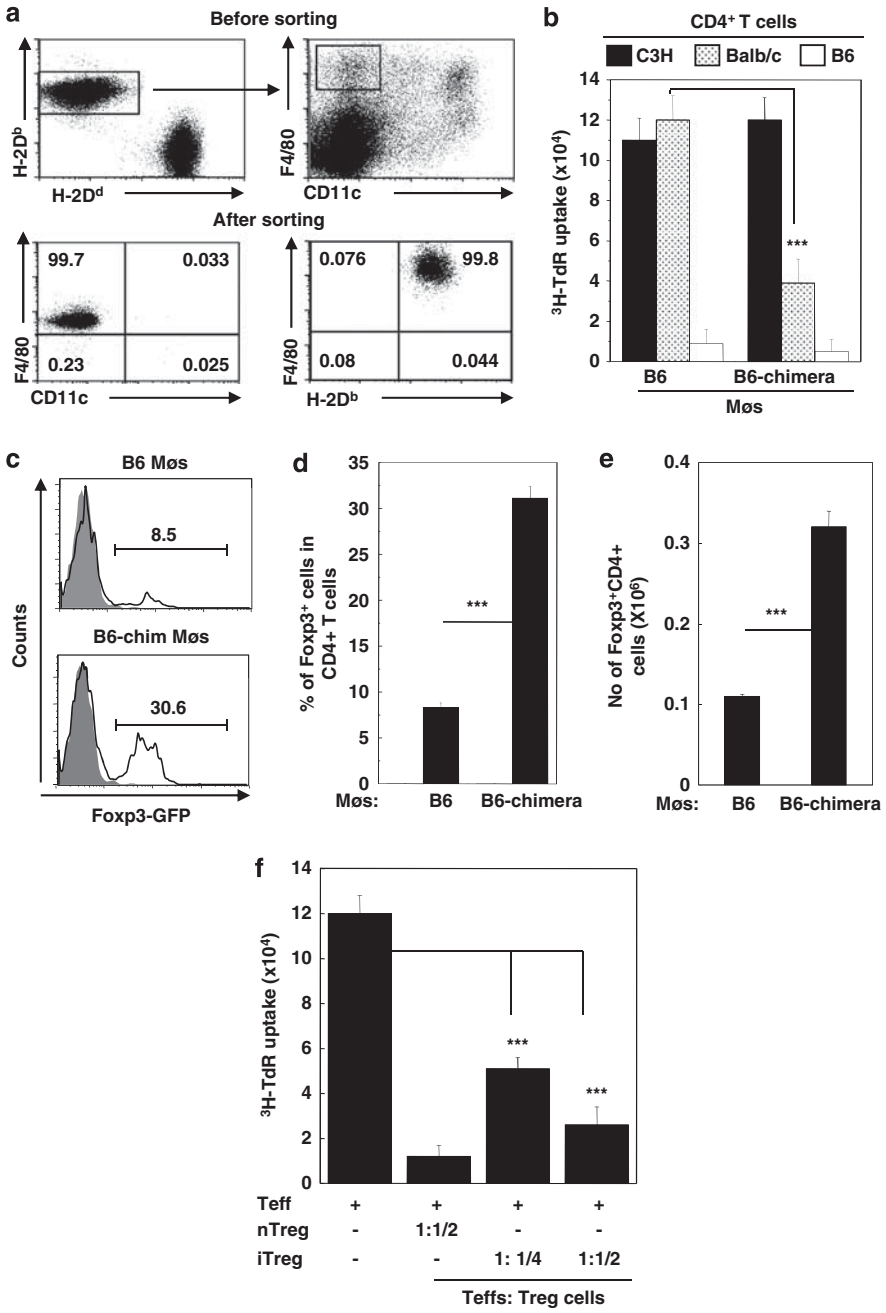

Figure 4 Donor F4/80$^+$CD11c$^-$ Møs from mixed chimera have the ability to induce the differentiation of recipient CD4$^+$Foxp3$^+$ T cells from CD4$^+$Foxp3GFP$^-$ T effector cells. The highly purified H-2D^{b+}F4/80$^-$CD11c$^-$ cells and allogeneic CD4$^+$Foxp3GFP$^-$ cells were sorted and co-cultured as described in Materials and methods. (**a**) The purity of the sorted H-2D^{b+}F4/80$^+$CD11c$^-$ cells. (**b**) Decreased immunogenicity to recipient BALB/c CD4$^+$Foxp3$^-$ T effector cells of purified B6 Møs isolated from mixed chimera. (**c**) One representative of FoxpGFP expression in gated CD4$^+$ T cells after allogeneic CD4$^+$Foxp3GFP$^-$ T cells were co-cultured with H-2D^{b+}F4/80$^+$CD11c$^-$ Møs isolated from control B6 or mixed chimera. (**d**) Mean percentages of CD4$^+$Foxp3GFP$^+$ T cells in CD4$^+$ T cells after CD4$^+$Foxp3GFP$^-$ T cells from naive BALB/c mice were co-cultured with H-2D^{b+}F4/80$^+$CD11c$^-$ Møs isolated from control B6 or mixed chimera. (**e**) Cell number of CD4$^+$Foxp3GFP$^+$ T cells after BALB/c CD4$^+$Foxp3GFP$^-$ T cells were co-cultured with H-2D^{b+}F4/80$^+$CD11c$^-$ Møs isolated from either control B6 or mixed chimera. (**f**) The immunosuppressive ability of CD4$^+$Foxp3GFP$^+$ T cells induced by H-2D^{b+}F4/80$^+$CD11c$^-$ Møs of mixed chimera. CD4$^+$Foxp3GFP$^-$ T effector cells from naive BALB/c mice were cultured with allogeneic B6 stimulators in the presence of isolated naive CD4$^+$Foxp3GFP$^+$ T cells (nTreg) or induced CD4$^+$Foxp3GFP$^+$ T cells (iTreg) by donor H-2D^{b+}F4/80$^+$CD11c$^-$ Møs. Data are shown as mean ± s.d. $^{***}P<0.001$ between the indicated groups.

when they were cultured with the third party C3H CD4$^+$ T cells (Supplementary Figure 4b).

Furthermore, the levels of TGF-β in the culture system in which naive recipient BALB/c, donor B6 or the third party C3H CD4$^+$ T cells were cultured with B6 Møs from either mixed chimeras or non-chimera controls were assayed by ELISA. As shown in Supplementary Figure 4c, significantly higher levels of TGF-β were detected when BALB/c T cells were cultured with B6 Møs of mixed chimeras compared with those cultured with B6 Møs from non-chimera controls ($P<0.01$). However, similar levels of TGF-β

were observed when C3H CD4$^+$ T cells were cultured with B6 Møs, regardless whether the later were from mixed chimeras or not.

Is IL-10 or/and TFG-β involved in the induction of recipient CD4$^+$ Treg cells by donor Møs of mixed chimeras? We next blocked IL-10 and/or TGF-β in the culture system by using anti-IL-10 or/and anti-TGF-β mAb to investigate their roles in the induction of recipient Treg cells by donor B6 Møs. Anti-IL-10mAb, but not anti-TGF-β mAb or control Ig, partially reversed the poor immunogenicity to recipient T cells of B6 Møs from mixed chimeras (Figure 5a). Importantly, anti-IL-10 significantly decreased the ability of donor B6 Møs separated from mixed chimeras to induce Treg cells as determined by the immunosuppressive assay ($P<0.001$; Figure 5b). These data indicate that the induction of recipient CD4$^+$Foxp3$^+$ Treg cells by donor Møs in mixed chimeras may be partially medicated by IL-10.

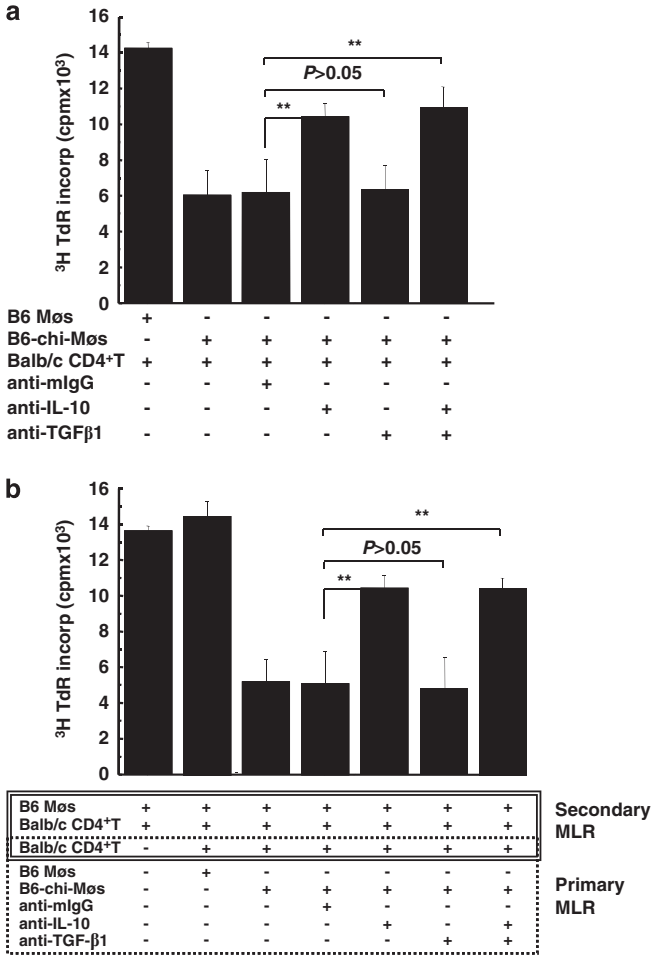

Figure 5 IL-10 but not TGF-β1 was involved in the induction of recipient CD4$^+$Foxp3$^+$ Treg cells by donor splenic Møs of mixed chimeras. (**a**) Blocking IL-10 but not TGF-β1 by mAb could partly reverse the poor proliferation response of CD4$^+$ T cells from naive recipient BALB/c mice to donor B6 splenic Møs of B6-to-BALB/c mixed chimeras. (**b**) Neutralizing IL-10 but not TGF-β1 by mAb could partly block the induction of Treg cells by donor splenic Møs of mixed chimeras. Anti-IL-10 and/or anti-TGF-β1 mAbs were added into the culture system in which naive recipient CD4$^+$ T cells were cultured with donor splenic Møs of mixed chimeras. The immunosuppressive ability of the induced Treg cells was determined by the ^{3}H-TdR incorporation assay. Data are shown as mean ± s.d. $^{**}P<0.01$ between the indicated groups. Data are representative of four independent experiments.

Recipient CD4$^+$Foxp3$^+$ Treg cells induced from CD4$^+$CD25$^-$ T cells by donor splenic Møs of mixed chimeras *in vivo*

To access the ability of donor Møs of mixed chimeras to induce recipient CD4$^+$ Treg cells *in vivo*, BALB/c CD4$^+$CD25$^-$ T cells with B6 Møs separated either from B6-to-BALB/c mixed chimeras or from B6 mice were adoptively transferred into severe combined immunodeficiency disease (SCID) mice. By 3–4 days after implantation, BALB/c CD4$^+$CD25$^-$ T cells expressed CD25, no matter whether they were injected with donor B6 Møs of mixed chimeras or non-chimeras (Figures 6A and B). However, significantly high percentages and cell numbers of CD4$^+$Foxp3$^+$ T cells and CD4$^+$CD25$^+$Foxp3$^+$ T cells were observed when BALB/c CD4$^+$CD25$^-$ T cells were co-injected with B6 Møs of mixed chimeras compared with those of mice with BALB/c CD4$^+$CD25$^-$ T cells and B6 splenic Møs of naive mice (Figures 6B and C). To confirm the immunosuppressive ability of recipient CD4$^+$ Treg cells induced by donor Møs of mixed chimeras *in vivo*, we observed their inhibition on the responses to alloantigens of BALB/c T effector cells using delayed-type hypersensitivity assays. BALB/c CD4$^+$CD25$^+$ T cells induced by donor B6 Møs of mixed chimeras showed similar inhibition on immunized BALB/c CD4$^+$CD25$^-$ T effector cells as freshly isolated CD4$^+$CD25$^+$ T cells, whereas BALB/c CD4$^+$CD25$^+$ T cells by B6 Møs of non-chimeras did not show significant inhibition on BALB/c CD4$^+$CD25$^-$ T cells (data not shown). These data collectively indicate that donor Møs of mixed chimeras have the ability to convert CD4$^+$CD25$^-$Foxp3$^-$ T effector cells into functional CD4$^+$Foxp3$^+$ Treg cells *in vivo*.

DISCUSSION

Immune tolerance to donor antigens can be achieved by mixed chimeras in which T, B and NK cell tolerant states have been well demonstrated.[10,17] In the present studies, donor Møs of mixed allogeneic chimeras show poor immunogenicity to recipient T cells whereas they have normal immunogenicity to the third party T cells, indicating the specificity of the altered immunogenicity in donor Møs in mixed chimeras. This specificity was further supported by the different cytokine products of Møs during co-culture with recipient T cells. Donor Møs of mixed chimeras produced more IL-10 and less IFN-γ when they were cultured with recipient T cells. The differential immune response of donor Møs to recipient or to the third party T cells may offer an important clue for the specific immune regulatory function or tolerant state of Møs in mixed chimeras. It is, to our knowledge, the first report that Møs displayed specific tolerance in mixed chimeras.

The mechanisms for the so-called 'specific tolerance' of donor Møs in a myeloablative mixed chimeric model are not clear at this moment. The possibility that this observation may be due to the contamination of DCs is unlikely as the highly purified donor F4/80$^+$CD11$^-$ cells (>99%) sorted from mixed chimeras showed similar results as those MACS-separated cells. The following possibilities may be involved: (1) Phenotype and functional adaptations and balance of different subsets of Møs. Functional plasticity of macrophages in distinct microenvironments has been reported in different models.[2,18,19] In the present study, donor Møs express significantly less MHC II, CD86 and CD40 molecules and produce less IFN-γ and more IL-10 when co-cultured with recipient T cells compared with those of naive mice, supporting the tolerogenic Møs phenotype alteration. (2) Educating the antigen-recognizing activation/inhibition receptors on Møs. A system of pattern recognition receptors has key role in antigen recognition of Møs in infections and transplant.[20–22] We hypothesized that the balance of these receptors may control the activation states of Møs. Møs in the chimeras lacking CD47 on non-hematopoietic cells

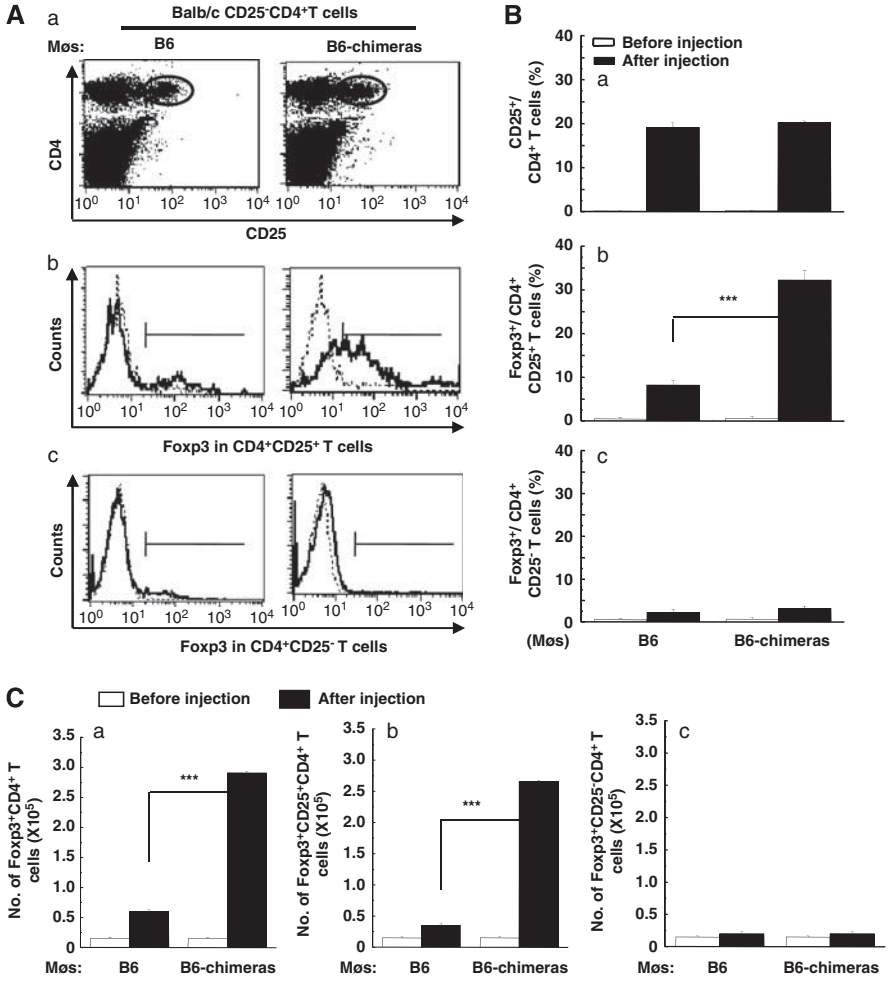

Figure 6 Induction of recipient CD4⁺Foxp3⁺ Treg cells from CD4⁺CD25⁻ T cells by donor splenic Møs of mixed chimeras *in vivo*. (**A**) Some BALB/c CD4⁺CD25⁻Foxp3⁻ T cells were converted into CD4⁺CD25⁺Foxp3⁺ T cells when CD4⁺CD25⁻ T cells from naive BALB/c mice were co-injected into syngeneic SCID mice with B6 splenic Møs of B6-to-BALB/c mixed chimeras but not with B6 splenic Møs of naive mice. One representative for the expression of CD25 on CD4⁺ T cells (a), the expression of Foxp3 in CD4⁺CD25⁺ T cells (b) and the expression of Foxp3 in CD4⁺CD25⁻ T cells (c) after adoptively transfer of BALB/c CD4⁺CD25⁻ T cells with B6 splenic Møs of mixed chimeras and naive mice were shown. (**B**) The mean percentages of CD25⁺ cells in CD4⁺ T cells (a), Foxp3⁺ cells in CD4⁺CD25⁺ T cells (b) and Foxp3⁺ cells in CD4⁺CD25⁻ T cells (c) after co-injection of CD4⁺CD25⁻ T cells and B6 splenic Møs of mixed chimeras and naive mice were summarized. (**C**) The total cell numbers of CD4⁺Foxp3⁺ T cells (a), CD4⁺CD25⁺Foxp3⁺ T cells (b) and CD4⁺CD25⁻Foxp3⁺ T cells (c) after co-injection of CD25⁻CD4⁺ T cells from naive BALB/c mice and B6 splenic Møs of mixed chimeras and naive mice were summarized. ***$P<0.001$ between the indicated groups. Six mice in each group were done. Data are representative of three independent experiments. Data are shown as mean ± s.d.

are tolerant to CD47(null) cells, indicating the potential importance of non-hematopoietic cells in the regulation of macrophage function.[21] (3) DCs can acquire intact allogeneic MHC molecules and promote T-cell response.[23–25] Whether it is the case for Møs-mediated immune unresponsiveness in mixed chimera needs to be determined.

Furthermore, donor Møs of mixed chimeras have the ability to induce recipient CD4⁺ Treg cells as indicated by the immunosuppression as well as the expression of Foxp3, a unique transcription factor in CD4⁺ Treg cells in mice.[11,26] Actually, the potential ability of Møs to induce Treg cells has been reported in infection models.[27,28] With the heterogeneity and plasticity, Møs may modify the phenotype, function and cytokine-secreting pattern of responding T cells in different microenvironments.[2,3,29,30] Donor Møs maturing in mixed allogeneic surroundings may be adaptive to this situation. This speculation was indirectly supported by the changes of donor Møs in respect of their phenotype and cytokine products. The *in vivo* effect of donor Møs in mixed chimeras to induce CD4⁺Foxp3⁺ Treg cells from recipient CD4⁺CD25⁻ T cells was studied in an SCID mouse model. Recipient CD4⁺CD25⁻ T cells could be converted into functional CD4⁺ Treg cells when recipient CD4⁺CD25⁻Foxp3⁻ T cells and donor Møs of mixed chimeras were adoptively transferred into syngeneic SCID mice. These data suggest that donor Møs of mixed chimeras have the ability to induce recipient CD4⁺Foxp3⁺ Treg cells *in vivo*.

Our further studies demonstrated that donor Møs of mixed chimera could induce the development of Foxp3⁺ Treg cells from CD4⁺CD25⁻Foxp3⁻ T effector cells. Interestingly, anti-IL-10 mAb but not anti-TGF-β1 mAb could partially but significantly block the induction of Treg cells by donor Møs from mixed chimeras, suggesting that the conversion of CD4⁺Foxp3⁺ Treg cells from CD4⁺Foxp3⁻ T cells may be achieved partly through an IL-10-dependent pathway

in this system. Although TGF-β was often related to the induction of Treg cells in various models,[31,32] the IL-10-mediated induction of Treg cells has also been reported[33,34] as we observed here. The poor involvement of TGF-β in the differentiation of Treg cells induced by donor Møs of mixed chimera may be due to the weakly enhanced TGF-β production in the present system.

It should be noted that the present study employed a myeloablative mixed chimeric model. Our preliminary study showed that donor Møs of full donor chimeras displayed similarly decreased immunogenicity to recipient T cells and had ability to induce $CD4^+Foxp3^+$ Treg cells *in vitro* (Liu GW and Zhao Y, unpublished data), indicating that non-hematopoietic cells may participate in the 'education' of donor Møs in a full donor chimeric model. Recently, some studies were nicely performed, showing that different T-cell tolerance mechanisms including clonal deletion and immunoregulation were involved in tolerance induction by different non-myeloablative conditioning regimens and even in the different stages of T-cell tolerance induction/maintenance in one model.[35,36] Whether those alterations of donor Møs will occur in clinically more relevant non-myeloablative mixed chimeras need to be determined. On the other hand, whether other innate immune cells such as DCs process similar alteration as Møs in mixed chimeras requests to be addressed.

In summary, donor Møs of mixed chimeras showed significantly decreased immunogenicity to recipient but not to the third party T cells. Importantly, donor Møs of mixed chimeras had ability to induce recipient $CD4^+$ T cells to be $CD4^+Foxp3^+$ Treg cells at least in part via an IL-10-dependent pathway. Thus, we observed an uncovered property or phoneme of Møs that Møs own the specific tolerance ability.

METHODS

Animals

Five-to-seven week-old C57BL/6 (B6), BALB/c, C3H/HeJ and SCID mice were purchased from Beijing University Experimental Animal Center (Beijing, China). All mice were maintained in a specific pathogen-free facility. All experimental manipulations were undertaken in accordance with the Institutional Guidelines for the Care and Use of Laboratory Animals.

Preparation of mixed allogeneic chimeras

B6-to-BALB/c mixed chimeras were prepared as previously described.[37] Briefly, BALB/c recipients received 8.0 Gy of total body irradiation and an i.v. injection of a mixture of T cell-depleted bone marrow cells from donor B6 (10×10^6 cells) and recipient BALB/c (10×10^6 cells) on day 0. Animals receiving such treatment showed about 50% of donor cells in all hematopoietic lineages for long term in the peripheral blood and spleens as determined by FCM (Becton Dickinson, San Jose, CA, USA).

Preparation of splenic Møs

Mouse splenic Møs were prepared as described previously.[38] Briefly, mouse splenocytes were adjusted to 5×10^6 cells ml^{-1} in RPMI 1640 medium (Gibco BRL, Grand Island, NY, USA) and cultured in 2% gelatin (Sigma) pre-treated 6-well plates (Costar, Cambridge, MA, USA) for 3–4 h at 37 °C and 5% CO_2. The non-adherent cells were removed by washing with warm RPMI 1640 medium. The adherent cells were harvested with 5 mM EDTA (Sigma) in ice-cold phosphate-buffered saline (pH 7.2) and adjusted to 1×10^6 cells ml^{-1}. Donor B6 Møs of mixed chimeras were separated by using negative selecting MACS with biotinylated anti-mouse H-$2D^d$ mAb (34-2-12) and BD IMag Streptavidin Particles-DM (BD Biosciences, San Diego, CA, USA). In some case, splenocytes harvested from either control B6 or mixed chimeric mice were incubated with anti-H-$2D^b$ anti-F4/80 and anti-CD11c mAb at 4 °C for 30 min. B6 Møs were sorted based on H-$2D^{b+}F4/80^+CD11c^-$ phenotype using a MoFlow (Beckman-Coulter, Fullerton, CA, USA). The purity of sorted B6 Møs (H-$2D^{b+}F4/80^+CD11c^-$) was above 99% as determined by FCM.

Immunofluorescence staining and FCM

The chimerism levels were determined as described previously.[3] Cells were stained with fluoresceine isothiocyanate (FITC)-labeled anti-CD3 mAb or anti-F4/80 mAb (BM8) versus phycoerythrin-labeled anti-H-$2D^b$ mAb (34-1-2S). Cells were analyzed using a FACScan (BD Biosciences, San Jose, CA, USA). To determine the intracellular expression of Treg cell-specific transcription factor Foxp3 in $CD4^+CD25^+$ cells, mouse splenocytes were first surface stained with Cy5-labeled anti-CD4 and FITC-labeled anti-CD25 mAbs as usual.[39] These cells were subsequently stained with phycoerythrin-labeled anti-mouse Foxp3 mAb (FJK-16s) or isotype control mAb, according to the manufacturer's protocol (eBiosciences, San Jose, CA, USA).

The IFN-γ, IL-10 or IL-12 productions in Møs of MLR were detected using BD Cytofix/Cytoperm plus (with GolgiPlug) intracellular staining kits (BD Biosciences, San Diego, CA, USA). $CD4^+$ T cells (2×10^6 cells per well) were co-cultured with allogeneic or syngeneic Møs (1×10^6 cells per well) in 6-well plates for 48 h. Cells were then pulsed with 1.0 μl ml^{-1} Brefeldin A (BD GolgiPlug; BD Biosciences) for the last 8 h of culture. The adherent cells were collected. After incubation with anti-FcR mAb (2.4G2), these cells were stained with FITC-conjugated anti-mouse F4/80 mAb (SK3), and then fixed and permeabilized with 500 μl of BD Cytofix/Cytoperm solution and then stained with 0.25 μg of anti-mouse IFN-γ mAb (GIR-208), anti-mouse IL-10 mAb (JES5-16E3) and anti-mouse IL-12 mAb (C15.6), respectively, according to the manufacturer's instructions.

Primary allogeneic MLR

$CD4^+$ T cells of naive BALB/c mice, except indicated in the Result section, were purified by negative selection of mouse splenocytes using mouse $CD4^+$ T-cell enrichment set-DM (BD Biosciences). MLR was performed and determined by ^{3}H-TdR incorporation as described previously.[38]

Detection of IL-2 and TGF-β1 levels in MLR by ELISA

$CD4^+$ T cells of naive recipient BALB/c or the third party C3H mice were cultured with allogeneic or syngeneic Møs in 96-well plates for 3–4 days. The levels of TGF-β1 and IL-2 in the supernatants were analyzed using ELISA kits (eBioscience, San Diego, CA, USA).

Induction of Treg cells *in vitro*

Sorted 1.5×10^6 $CD4^+$ T cells or $CD4^+CD25^-$ T cells of naive BALB/c or C3H mice as responder cells were cultured with 1.5×10^6 donor B6 Møs separated from either B6 or mixed B6-to-BALB/c chimeric mice in a total volume of 2 ml of RPMI 1640 medium containing 10% (vol/vol) mouse serum in 6-well tissue culture plates for 4–5 days. For induction of Treg cells from $CD4^+CD45RB^{high}GFP^-$ T cells isolated from Foxp3GFP-knockin mice (BALB/c background) by Møs, 1.0×10^6 sorted $CD4^+CD45RB^{high}Foxp3GFP^-$ T cells were cultured with 1.0×10^6 sorted B6 $F4/80^+CD11c^-$ Møs of either control B6 or B6-to-BALB/c mixed chimeric mice for 4–5 days. The percentage of $Foxp3gfp^+$ cells in $CD4^+$ cells was detected by FCM.

Immunosuppression assay of Treg cells

Enriched $CD4^+CD25^+$ T cells were achieved using a $CD4^+CD25^+$ Treg Cells Isolation Kit with MidiMACS Separator according to the manufacturer's protocols (Miltenyi, Bergisch Gladbach, Germany) or sorted by a FACSAria flow cytometer (BD, San Jose, CA, USA).[40,41] The immunosuppressive ability on the proliferation of T effector cells of separated $CD4^+CD25^+$ T cells and induced Treg cells was done as described previously.[42] In some experiments, the induced $CD4^+Foxp3GFP^+$ T cells were sorted and Treg suppressive function assay was performed as described above.

Induction of recipient Treg cells by donor Møs of mixed chimeras *in vivo*

$CD4^+CD25^-$ T-cell population of naive BALB/c mice was sorted by a FACSAria flow cytometer (BD, San Jose, CA, USA). In all, 6×10^6 B6 Møs separated from either control or mixed chimeric mice with 6×10^6 BALB/c $CD4^+CD25^-$ T cells were injected into SCID mice through tail vein, respectively. By 3–4 days after injection, the phenotype and immunosuppressive ability of $CD4^+CD25^+$ T cells were assessed, as reported previously.[40]

Statistical analysis

All data are presented as the mean ± s.d. Statistical analyses were performed using the Student's unpaired *t*-test. A *P*-value <0.05 was considered to be statistically significant.

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1 Smith PD, Ochsenbauer-Jambor C, Smythies LE. Intestinal macrophages: unique effector cells of the innate immune system. *Immunol Rev* 2005; **206**: 149–159.
2 Stout RD, Suttles J. Functional plasticity of macrophages: reversible adaptation to changing microenvironments. *J Leuk Biol* 2004; **76**: 509–513.
3 Stout RD, Suttles J. Immunosenescence and macrophage functional plasticity: dysregulation of macrophage function by age-associated microenvironmental changes. *Immunol Rev* 2005; **205**: 60–71.
4 Goerdt S, Politz O, Schledzewski K, Birk R, Gratchev A, Guillot P *et al.* Alternative versus classical activation of macrophages. *Pathobiology* 1999; **67**: 222–226.
5 Jose MD, Ikezumi Y, van Rooijen N, Atkins RC, Chadban SJ. Macrophages act as effectors of tissue damage in acute renal allograft rejection. *Transplantation* 2003; **76**: 1015–1022.
6 Wyburn KR, Jose MD, Wu H, Atkins RC, Chadban SJ. The role of macrophages in allograft rejection. *Transplantation* 2005; **80**: 1641–1647.
7 Fox A, Mountford J, Braakhuis A, Harrison LC. Innate and adaptive immune responses to nonvascular xenografts: evidence that macrophages are direct effectors of xenograft rejection. *J Immunol* 2001; **166**: 2133–2140.
8 Cantu E, Gaca JG, Palestrant D, Baig K, Lukes DJ, Gibson SE *et al.* Depletion of pulmonary intravascular macrophages prevents hyperacute pulmonary xenograft dysfunction. *Transplantation* 2006; **81**: 1157–1164.
9 Yi S, Hawthorne WJ, Lehnert AM, Ha H, Wong JK, van Rooijen N *et al.* T cell-activated macrophages are capable of both recognition rejection of pancreatic islet xenografts. *J Immunol* 2003; **170**: 2750–2758.
10 Sykes M. Mixed chimerism and transplant tolerance. *Immunity* 2001; **14**: 417–424.
11 Hori S, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor Foxp3. *Science* 2003; **299**: 1057–1061.
12 Bharat A, Fields RC, Steward N, Trulock EP, Patterson GA, Mohanakumar T. CD4+25+ regulatory T cells limit Th1-autoimmunity by inducing IL-10 producing T cells following human lung transplantation. *Am J Transplant* 2006; **6**: 1799–1808.
13 Lin HH, Faunce DE, Stacey M, Terajewicz A, Nakamura T, Zhang-Hoover J *et al.* The macrophage F4/80 receptor is required for the induction of antigen-specific efferent regulatory T cells in peripheral tolerance. *J Exp Med* 2005; **201**: 1615–1625.
14 Liu G, Ma H, Jiang L, Peng J, Zhao Y. The immunity of splenic and peritoneal F4/80(+) resident macrophages in mouse mixed allogeneic chimeras. *J Mol Med* 2007; **85**: 1125–1135.
15 Sakaguchi S, Ono M, Setoguchi R, Yagi H, Hori S, Fehervari Z *et al.* Foxp3+ CD25+ CD4+ natural regulatory T cells in dominant self-tolerance and autoimmune disease. *Immunol Rev* 2006; **212**: 8–27.
16 Yi H, Zhen Y, Jiang L, Zheng J, Zhao Y. The phenotypic characterization of naturally occurring regulatory CD4+CD25+ T cells. *Cell Mol Immunol* 2006; **3**: 189–195.
17 Zhao Y, Ohdan H, Manilay JO, Sykes M. NK cell tolerance in mixed allogeneic chimeras. *J Immunol* 2003; **170**: 5398–5405.
18 Mantovani A, Sozzani S, Locati M, Allavena P, Sica A. Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. *Trends Immunol* 2002; **23**: 549–555.
19 Schenk M, Mueller C. Adaptations of intestinal macrophages to an antigen-rich environment. *Semin Immunol* 2007; **19**: 84–93.
20 Palm NW, Medzhitov R. Pattern recognition receptors and control of adaptive immunity. *Immunol Rev* 2009; **227**: 221–233.
21 Blazar BR, Lindberg FP, Ingulli E, Panoskaltsis-Mortari A, Oldenborg PA, Iizuka K *et al.* CD47 (integrin-associated protein) engagement of dendritic cell and macrophage counterreceptors is required to prevent the clearance of donor lymphohematopoietic cells. *J Exp Med* 2001; **194**: 541–549.
22 Ide K, Wang H, Tahara H, Liu J, Wang X, Asahara T *et al.* Role for CD47-SIRPalpha signaling in xenograft rejection by macrophages. *Proc Natl Acad Sci USA* 2007; **104**: 5062–5066.
23 Herrera OB, Golshayan D, Tibbott R, Salcido Ochoa F, James MJ, Marelli-Berg FM, Lechler RI. A novel pathway of alloantigen presentation by dendritic cells. *J Immunol* 2004; **173**: 4828–4837.
24 Russo V, Tanzarella S, Dalerba P, Rigatti D, Rovere P, Villa A *et al.* Dendritic cells acquire the MAGE-3 human tumor antigen from apoptotic cells and induce a class I-restricted T cell response. *Proc Natl Acad Sci USA* 2000; **97**: 2185–2190.
25 Harshyne LA, Watkins SC, Gambotto A, Barratt-Boyes SM. Dendritic cells acquire antigens from live cells for cross-presentation to CTL. *J Immunol* 2001; **166**: 3717–3723.
26 Fontenot JD, Gavin MA, Rudensky AY. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. *Nat Immunol* 2003; **4**: 330–336.
27 Taams LS, Akbar AN. Peripheral generation and function of CD4+CD25+ regulatory T cells. *Curr Top Microbiol Immunol* 2005; **293**: 115–131.
28 Terrazas LI, Montero D, Terrazas CA, Reyes JL, Rodriguez-Sosa M. Role of the programmed Death-1 pathway in the suppressive activity of alternatively activated macrophages in experimental cysticercosis. *Int J Parasitol* 2005; **35**: 1349–1358.
29 Gordon S, Taylor PR. Monocyte and macrophage heterogeneity. *Nat Rev Immunol* 2005; **5**: 953–964.
30 Kurtz J, Wekerle T, Sykes M. Tolerance in mixed chimerism - a role for regulatory cells? *Trends Immunol* 2004; **25**: 518–523.
31 Chen W, Jin W, Hardegen N, Lei KJ, Li L, Marinos N *et al.* Conversion of peripheral CD4+CD25- naive T cells to CD4+CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3. *J Exp Med* 2003; **198**: 1875–1886.
32 Zhang L, Yi H, Xia XP, Zhao Y. Transforming growth factor-beta: an important role in CD4+CD25+ regulatory T cells and immune tolerance. *Autoimmunity* 2006; **39**: 269–276.
33 Ghoreishi M, Dutz JP. Tolerance induction by transcutaneous immunization through ultraviolet-irradiated skin is transferable through CD4+CD25+ T regulatory cells and is dependent on host-derived IL-10. *J Immunol* 2006; **176**: 2635–2644.
34 Lan YY, Wang Z, Raimondi G, Wu W, Colvin BL, de Creus A *et al.* Alternatively activated" dendritic cells preferentially secrete IL-10, expand Foxp3+CD4+ T cells, and induce long-term organ allograft survival in combination with CTLA4-Ig. *J Immunol* 2006; **177**: 5868–5877.
35 Domenig C, Sanchez-Fueyo A, Kurtz J, Alexopoulos SP, Mariat C, Sykes M *et al.* Roles of deletion and regulation in creating mixed chimerism and allograft tolerance using a nonlymphoablative irradiation-free protocol. *J Immunol* 2005; **175**: 51–60.
36 Bigenzahn S, Blaha P, Koporc Z, Pree I, Selzer E, Bergmeister H *et al.* The role of non-deletional tolerance mechanisms in a murine model of mixed chimerism with costimulation blockade. *Am J Transplant* 2005; **5**: 1237–1247.
37 Ildstad ST, Wren SM, Bluestone JA, Barbieri SA, Sachs DH. Characterization of mixed allogeneic chimeras. Immunocompetence, *in vitro* reactivity, and genetic specificity of tolerance. *J Exp Med* 1985; **162**: 231–244.
38 Liu G, Xia XP, Gong SL, Zhao Y. The macrophage heterogeneity: difference between mouse peritoneal exudate and splenic F4/80+ macrophages. *J Cell Physiol* 2006; **209**: 341–352.
39 Zhang A, Qu Y, Zhang B, Zhang L, Zeng C, Peng J, Ji X *et al.* The different effects of indirubin on effector and CD4+CD25+ regulatory T cells in mice: potential implication for the treatment of autoimmune diseases. *J Mol Med* 2007; **85**: 1263–1270.
40 Sun Z, Zhao L, Wang H, Sun L, Yi H, Zhao Y. Presence of functional mouse regulatory CD4+CD25+T cells in xenogeneic neonatal porcine thymus-grafted athymic mice. *Am J Transplant* 2006; **6**: 2841–2850.
41 Qu Y, Zhang B, Zhao L, Liu G, Ma H, Rao E *et al.* The effect of immunosuppressive drug rapamycin on regulatory CD4+CD25+Foxp3+T cells in mice. *Transpl Immunol* 2007; **17**: 153–161.
42 Wang H, Zhao L, Sun Z, Sun L, Zhang B, Zhao Y. A potential side effect of cyclosporin A: inhibition of CD4+CD25+ regulatory T cells in mice. *Transplantation* 2006; **82**: 1484–1492.

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